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(21) 出願番号	特願平4-270684	(71) 出願人	000109015 ダイナボット株式会社 東京都港区虎ノ門3-8-21 第33森ビル
(22) 出願日	平成4年(1992)8月28日	(72) 発明者	武井 俊憲 千葉県松戸市稔台344 ダイナボット株式 会社総合研究所内
		(72) 発明者	井上 裕三 千葉県松戸市稔台344 ダイナボット株式 会社総合研究所内
		(72) 発明者	朝比奈 亜紀 千葉県松戸市稔台344 ダイナボット株式 会社総合研究所内
		(74) 代理人	弁理士 斉藤 武彦 (外1名) 最終頁に続く

(54) 【発明の名称】 抗体測定用試薬

(57) 【要約】

【目的】 感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドを用いる試薬による免疫学的方法による検体中の抗体測定系で、抗体を試薬の貯蔵状態などに影響されることなく高い感度で測定すると共ににより正確に測定する。

【構成】 検体中の抗体を免疫学的方法により測定するための感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチド含有試薬において、該試薬に還元剤を含有せしめるか、固相化された該感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチド含有試薬を還元剤で処理することにより抗体を試薬の貯蔵状態などに影響されることなく高い感度で測定すると共ににより正確に測定できる。

【効果】 検体中の抗体を免疫学的方法により測定するにさいし、その試薬の感度を高めることが出来る。

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【特許請求の範囲】

【請求項1】 検体中の抗体を免疫学的方法により測定するための感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチド含有試薬において、該試薬に還元剤を含有せしめるか、固相化された該感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチド含有試薬を還元剤で処理することを特徴とする抗体測定用試薬。

【請求項2】 該抗原がHCV抗原またはそれと実質的に同等の作用を有するペプチドである請求項1記載の抗体測定用試薬。

【請求項3】 還元剤が反応用溶媒中に存在する請求項1記載の抗体測定用試薬。

【請求項4】 該試薬が、HCV抗原またはそれと実質的に同等の作用を有するペプチドを担体に固相化した抗原であり、該試薬が還元剤で処理されている請求項1記載の抗体測定用試薬。

【請求項5】 該抗原がHCVゲノム上の非構造領域のNS3領域である請求項2または4記載の抗体測定用試薬。

【請求項6】 該抗原が遺伝子組換え技術による発現産物である請求項5記載の抗体測定用試薬。

【請求項7】 該抗原が合成ペプチドである請求項5記載の抗体測定用試薬

【請求項8】 該試薬が、担体を含み、該担体がビーズ、チューブ、プレート、赤血球、またはラテックス粒子である請求項1記載の抗体測定用試薬。

【請求項9】 還元剤がチオール基の酸化防止剤である請求項1～8記載の抗体測定用試薬。

【請求項10】 還元剤がジチオスレイトール、ジチオエリスリトール、チオグリコール酸、システイン、グルタチオン、2-メルカプトエタノール、2-メルカプトエチルアミンおよびこれらの混合物から成る群より選ばれた少なくとも一つである請求項1～8記載の抗体測定用試薬。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は抗体を測定するための試薬、特に免疫学的方法により検体中の抗体を感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドを用いて測定するための試薬において、抗体を試薬の貯蔵状態などに影響されることなく高い感度で測定すると共により正確に測定するための測定用試薬に関する。特に、本発明はC型肝炎ウイルス(HCV)に対する抗体を測定するための試薬、特に免疫学的方法により検体中のHCV抗体を高い感度で測定すると共により正確に測定するための測定用試薬に関する。

【0002】

【従来の技術】C型肝炎ウイルス(HCV)による感染を診断する方法としては、1988年に米国カイロン社

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✓

よりHCVゲノム上の非構造領域であるNS3およびNS4領域にコードされるC100-3抗原を使用したHCV抗体測定系が開発された。1991年には、HCVゲノム上の構造領域であるコア領域およびC100-3抗原とは重複しないNS3領域にコードされるコア抗原および33C抗原の使用により更に感度および検出率の優れたHCV抗体測定系が開発された。これらHCV抗体の測定法としては、赤血球やラテックス粒子を抗原担持担体として用いる凝集法、ビーズ、チューブ、あるいはプレートを抗原固相化担体として用いるイムノメトリック法等が用いられている。しかし、抗原を担体上へ固相化する工程中あるいは調製された試薬の保存中に抗原の活性が反応溶液中で急激に低下し、抗原抗体反応が十分に進行できないために測定感度が十分に上がらない、更には抗原の活性が経時的に変化するために感度の再現性が悪化する等の問題があった。このようにある種の抗原を用いた試薬の場合、試薬の保存中に抗原の活性が反応溶液中で急激に低下し、抗原抗体反応が十分に進行できず測定感度が十分に上がらないとか、抗原の活性が経時的に変化するために感度の再現性が悪化する等の問題があった。

【0003】

【発明が解決しようとする課題】本発明者等は、従来のHCV抗体測定系における感度の問題を解決すべく鋭意研究した結果、この感度不良の問題は、HCV抗原、特にHCVゲノム上のNS3領域にコードされているタンパク質に含まれているシステインが自然酸化を受け、ジスルフィド結合等になることに起因することを解明した。本発明者等は、これらの知見に基づきHCV測定系に還元剤、特にチオール保護剤を添加することにより、そのHCV抗体測定系の感度の低下の問題を防止でき、さらにその還元剤処理は該測定系に悪影響を与えないものであることを知った。本発明者等は、この知見から感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドを用いる試薬による免疫学的方法による検体中の抗体測定系にも還元剤、特にチオール保護剤を添加したりすることにより感度の低下を防止でき、ただでなく、該測定系に悪影響を与えないようになしうるとの応用ができると考え、本発明を完成したものである。

【0004】

【課題を解決するための手段】本発明は検体中の抗体を免疫学的方法により測定するための感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチド含有試薬において、還元剤を含有せしめること、あるいは担体に固相化した抗原を還元剤で処理することを特徴とし、測定系の感度を高めたり、測定結果の信頼性を高めるにある。特に本発明は検体中のHCV抗体を免疫学的方法により測定するための試薬において、該HCV抗原またはそれと実質的に同等の作用を有するペプ

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チド含有試薬に還元剤を含有せしめること、あるいは担体に固相化したHCV抗原またはそれと実質的に同等の作用を有するペプチドを還元剤で処理することを特徴とし、測定系の感度を高めたり、測定結果の信頼性を高めるにある。本発明において用いられる検体としては、問題の抗体を含有する生体由来成分であればよく、例えば、血液、血清、精液、脊髄液、リンパ液、痰、涙、唾液、乳汁、白血球、消化器官粘液、尿等の体液あるいは組織液等が挙げられ、更にインビトロの細胞培養液等が挙げられるが、これらに限定されるものではない。

【0005】本発明は、赤血球やラテックス粒子等の微粒子担体に感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドを感作した感作担体を用いて、凝集反応により検体中の特異抗体を免疫学的に測定するために使用する試薬を製造するに際し、担体にその抗原を感作した後、得られた感作担体を還元剤を含有する緩衝液に分散させ、上記感作担体含有緩衝液を凍結乾燥することにより達成することが出来る。特に本発明は、赤血球やラテックス粒子等の微粒子担体にHCV抗原を感作した感作担体を用いて、凝集反応により検体中のHCV抗体を免疫学的に測定するために使用する試薬を製造するに際し、担体にHCV抗原を感作した後、得られた感作担体を還元剤を含有する緩衝液に分散させ、上記感作担体含有緩衝液を凍結乾燥することからなる。ここで用いることが出来る還元剤としては、チオール保護剤として知られたものが挙げられる。また、還元剤としては、チオール基の酸化防止剤であるものが好ましく、例えばジチオスレイトール、ジチオエリスリトール、チオグリコール酸、システイン、グルタチオン、2-メルカプトエタノール、2-メルカプトエチルアミンおよびこれらの混合物から成る群より選ばれた少なくとも一つであるものが挙げられる。特に、ジチオスレイトール、グルタチオン、2-メルカプトエタノール等が好ましい。

【0006】ここで用いることが出来る微粒子担体としては、微粒子担体として広く知られたものが挙げられ、例えば合成樹脂、ニトロセルロース等の天然あるいは合成の高分子等から作られたものの他、ラテックス粒子等あるいは赤血球等が挙げられる。本発明に従えば、凝集反応時に感作担体を懸濁させる際の使用緩衝液中に上記還元剤を添加してもよい。また、本発明は、感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドを不溶性担体に固相化することによって得られた固相化抗原を用いて、エンザイムイムノアッセイ(EIA)、ラジオイムノアッセイ(RIA)、あるいはフルオロイムノアッセイ(FIA)等の方法により検体中の特異抗体を免疫学的に測定するために使用する試薬を製造するに際し、不溶性担体に感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドを固相化した後、得られた抗原結合固相を前記

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の還元剤あるいはこれらの混合物を含有する緩衝液に浸漬した後、上記固相を乾燥することにより達成することが出来る。特に、本発明では、HCV抗原を不溶性担体に固相化することによって得られた固相化抗原を用いて、エンザイムイムノアッセイ(EIA)、ラジオイムノアッセイ(RIA)、あるいはフルオロイムノアッセイ(FIA)等の方法により検体中のHCV抗体を免疫学的に測定するために使用する試薬を製造するに際し、不溶性担体にHCV抗原を固相化した後、得られた抗原結合固相を前記の還元剤あるいはこれらの混合物を含有する緩衝液に浸漬した後、上記固相を乾燥することにより達成することが出来る。

【0007】ここで用いることが出来る還元剤としては、上記と同様なチオール保護剤として知られたものが挙げられる。本発明に従えば、抗原結合固相に検体中の特異抗体を反応させる際に使用する反応液中に上記の還元剤あるいはこれらの混合物を添加してもよい。ここで用いることが出来る不溶性担体としては、固相化用担体として広く知られたものが挙げられ、例えばポリエチレン、ポリプロピレン、ポリスチレン、ポリアクリレート等の合成樹脂、ニトロセルロース、重合アミノ酸、多糖等の天然あるいは合成の高分子、ガラス等から作られた、粒子、膜、ビーズ、チューブ、あるいはプレート等の形状のものがある。また、固相化にあたっては、物理的吸着法あるいは化学的に結合剤を用いて固相化せしめる方法がある。化学的な結合剤としては、通常の当業者に知られたものの中から選択することができるが、例えば、6-マレイミドカプロン酸、2-プロモ酢酸、2-ヨード酢酸、コハク酸等の活性エステル、トリアジンの活性エステル、スルホン酸エステル誘導体等が挙げられるが、これらに限定されるものではない。

【0008】本発明で用いる抗原としては、感受性チオール基を持つ抗原またはそれと実質的に同等の作用を有するペプチドが挙げられる。ここで感受性チオール基とはタンパク質またはペプチドに含まれているシステイン中のチオール基であって、通常のもので自然酸化または人工的酸化に感受性で、該抗原の活性に大きく影響を与えうる基をいう。ここで抗原の活性とは、特異的な抗原抗体反応をなすことができるものをいい、特に検体中の特異抗体と免疫学的方法で反応する特異抗原の活性を指す。本発明で用いる抗原は、このように感受性チオール基を持つものであれば、遺伝子工学的的手法によって作製された発現産物たる組換え抗原、あるいは合成ペプチドであるものも格別の制限無く用いることが出来る。本発明においては、たとえ分子中にシステインのチオール基またはそれに由来するジスルフィド結合を有していてもそれらが該抗原の活性に影響を与えない場合は、本発明で還元剤による処理をなす抗原としてはそれを意図しない。特に本発明で用いるHCV抗原は、遺伝子工学的的手法によって作製された発現産物たる組換えH

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CV抗原、あるいは合成ペプチドであるHCV抗原ペプチドが挙げられる。本発明で用いるHCV抗原として、好ましいものとしてはHCVゲノム上の非構造領域のNS3領域に相当するものが挙げられる。

【0009】

【実施例】以下に実施例を挙げて、本発明を更に具体的に説明する。

実施例1 DTT添加による感作血球への影響

ヒト固定化赤血球をリン酸緩衝液(pH 7.4)で3回洗浄後、pH5.7の酢酸緩衝液に1容量%となるように懸濁し、遺伝子組換え技術により産生された精製HCV抗原(コア抗原、33C抗原およびC100抗原の混合物)を最終濃度が6 μ g/mlとなるように添加し、温室で1時間攪拌した後に、リン酸緩衝液(pH 7.4)で3回洗浄し、7.5%のサツカロースを含有*

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*するリン酸緩衝液(pH 7.4)中で凍結乾燥して、HCV抗原感作赤血球を調製した。得られたHCV抗原感作赤血球を2mMのジチオスレイトール(DTT)を含むトリス塩酸緩衝液(pH 7.8)及び対照としてDTTを含まないトリス塩酸緩衝液に、1容量%となるように再懸濁を行い、感度を比較した。感度の比較は、HCV抗体陽性ヒト血清を同抗体が陰性である血清により予め段階希釈したものを感度管理用血清として使用した。マイクロタイタープレートの各ウエルに、リン酸緩衝液(pH 7.4)25 μ l、及び各濃度の感度管理用血清25 μ lを分注し、さらに感作血球を25 μ l分注して、ミキサーで30秒間攪拌し、室温で1時間放置後、結果を目視により判定した。

【表1】

	感作血球懸濁液	
	DDT存在	DDT非存在
感度管理用血清の希釈率		
血清A		
× 1	+	+
× 2	+	+
× 4	+	+
× 8	+	-
× 16	-	-
× 32	-	-
× 64	-	-
血清B		
× 1	+	+
× 2	+	+
× 4	+	+
× 8	+	-
× 16	-	-
× 32	-	-
× 64	-	-

(+ : 陽性、- : 陰性)

表1の結果より、DTTを含有する緩衝液に感作血球を再懸濁した場合には感度が上昇していることが示された。

【0010】

実施例2 2-ME添加による感作血球への影響

実施例1と同様に調製した感作血球を40mMの2-メルカプトエタノール(2-ME)を含有するトリス塩酸

緩衝液(pH 7.8)に1容量%となるように再懸濁し、懸濁後の保存安定性について2-MEを含まないトリス塩酸緩衝液を対照にして比較した。溶解後の保存は2~8℃保存とし、感作血球の感度比較は実施例1に準じて行った。

【表2】

2～8℃ における 保存期間 (日)	感作血球懸濁液					
	2-ME存在			2-ME非存在		
	0	7	14	0	7	14
感度管理用 血清の希釈率						
血清A						
× 1	+	+	+	+	+	+
× 2	+	+	+	+	+	—
× 4	+	+	+	+	—	—
× 8	+	+	+	+	—	—
× 16	—	—	—	—	—	—
× 32	—	—	—	—	—	—
× 64	—	—	—	—	—	—
血清B						
× 1	+	+	+	+	+	+
× 2	+	+	+	+	+	—
× 4	+	+	+	+	—	—
× 8	+	+	+	+	—	—
× 16	—	—	—	—	—	—
× 32	—	—	—	—	—	—
× 64	—	—	—	—	—	—

(+ : 陽性、- : 陰性)

表2の結果より、2-MEの添加により、感作血球の懸濁後の保存安定性が改善されたことが確認された。

【0011】

実施例3 グルタチオン添加による感作血球への影響

実施例1に準じてHCV抗原を感作させた血球を、7.5%サツカロース含有リン酸緩衝液(pH 7.4)に最終濃度が40mMとなるようにグルタチオン(GSH)を添加した凍結乾燥用の緩衝液中で凍結乾燥し、感

作血球とした。対照としてGHSを含まない緩衝液中で凍結乾燥し、対照感作血球とした。感作血球及び対照感作血球をトリス塩酸緩衝液(pH 7.8)に1容量%となるように懸濁し、室温における懸濁後の安定性について、感作血球の感度により比較した。感度比較は実施例1に準じて行った。

【表3】

室温での 保存期間 (日)	凍結乾燥用緩衝液					
	GSH存在			GSH非存在		
	0	1	2	0	1	2
感度管理用 血清の希釈率						
血清A						
× 1	+	+	+	+	+	+
× 2	+	+	+	+	+	-
× 4	+	+	+	+	-	-
× 8	+	+	+	+	-	-
× 16	-	-	-	-	-	-
× 32	-	-	-	-	-	-
× 64	-	-	-	-	-	-
血清B						
× 1	+	+	+	+	+	-
× 2	+	+	+	+	+	-
× 4	+	+	+	+	-	-
× 8	+	+	+	+	-	-
× 16	-	-	-	-	-	-
× 32	-	-	-	-	-	-
× 64	-	-	-	-	-	-

(+: 陽性、-: 陰性)

表3の結果より、GSHを添加して凍結乾燥を行った感作血球は、懸濁後の安定性が良いことが確認された。

【0012】

実施例4 2-ME添加による固相化抗原への影響

1/4インチのポリスチレンビーズを7.5%のサツカロースを含有するリン酸緩衝液(pH 7.4)中に入れ、実施例1で使用したHCV抗原を最終濃度が10μl/mlとなるように添加し、37℃で1時間静置した後、リン酸緩衝液(pH 7.4)で3回洗浄し、さらに7.5%のサツカロースを含有するリン酸緩衝液(pH 7.4)中に浸漬した後、乾燥し、HCV抗原固相化ビーズを得た。上記HCV抗原固相化ビーズ1個を、反应用緩衝液として20mMの2-MEを含有するトリ

30

ス塩酸緩衝液(pH 7.8)200μlを入れたワイドウエルのトレイにいれ、室温で1時間放置後、感度管理用血清10μlを添加し、37℃で1時間反応させた。反応終了後、生理食塩水で3回洗浄し、ペルオキシダーゼ標識抗ヒトイムノグロブリンG抗体200μlを添加し、37℃で30分反応させた。反応終了後に生理食塩水で3回洗浄し、オルトエチレンジアミン及び過酸化水素を含む基質溶液を1ml加え、室温で30分反応後に490nmの吸光度を測定することにより判定した。比較対照のために、2-MEを含有しないトリス塩酸緩衝液を反应用緩衝液として用いて同様の操作を行い、判定した。

【表4】

測定用緩衝液				
2-ME存在			2-ME非存在	
	吸光度	判定	吸光度	判定
感度管理用血清の希釈率				
血清A				
× 1	1. 8 2	+	0. 6 9	+
× 2	1. 4 4	+	0. 4 5	+
× 4	0. 9 6	+	0. 3 2	-
× 8	0. 5 6	+	0. 2 6	-
血清B				
× 1	1. 2 9	+	1. 3 1	+
× 2	0. 9 3	+	0. 7 3	+
× 4	0. 5 9	+	0. 4 7	-
× 8	0. 3 8	-	0. 3 0	-
× 16	0. 2 5	-	0. 2 6	-

(+: 陽性、-: 陰性)

表4の結果より、2-MEを反応用緩衝液に添加することにより、感度の上昇が見られた。

【0013】実施例5 2-MEの添加による影響
実施例1に準じて調製したHCV抗原感作血球をトリス塩酸緩衝液(pH7.8)に懸濁し、2~8℃で2週間放置後、HCV抗体陽性検体として抗コア(HCVのコ*

*ア領域)、抗33C(HCVのNS3領域)及び抗C100(HCVのNS3~NS4領域)のそれぞれの抗体を主に含む検体3種に対する感作血球の感度を測定し、実施例1に準じて検討した。懸濁液に40mMの2-MEを添加し、同様に感度を検討した。

【表5】

懸濁液に2-MEを 添加した感作血球		懸濁液に2-MEを 添加しない感作血球	
検体の希釈率			
抗33C抗体			
×	128	+	+
×	256	+	+
×	512	+	-
×	1024	+	-
×	2048	+	-
×	4096	+	-
×	8192	-	-

(+: 陽性、-: 陰性)

その結果より、2週間の放置により感度の低下した感作血球の感度が、HCVゲノム上のNS3領域にコードされる33C抗原では、2-MEの添加により回復しているのが認められた。

【0014】

【発明の効果】感受性チオール基を持つ抗原またはそれ

と実質的に同等の作用を有するペプチド含有抗体測定用試薬に還元剤を含有せしめるか、固相化された該試薬を還元剤で処理して、検体中の抗体を免疫学的方法により測定するにさいし、その試薬の感度を高めることが出来る。

フロントページの続き

(72)発明者 時田 進
千葉県松戸市稔台344 ダイナボット株式
会社総合研究所内

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Entry 1 of 2

File: JPAB

Mar 18, 1994

PUB-NO: JP406074956A

DOCUMENT-IDENTIFIER: JP 06074956 A

TITLE: REAGENT FOR ANTIBODY MEASUREMENT

PUBN-DATE: March 18, 1994

INVENTOR-INFORMATION:

NAME

TAKEI, TOSHINORI

INOUE, YUZO

ASAHINA, AKI

TOKITA, SUSUMU

ASSIGNEE-INFORMATION:

NAME

COUNTRY

DAINABOTSUTO KK N/A

APPL-NO: JP04270684

APPL-DATE: August 28, 1992

INT-CL (IPC): G01N 33/576; G01N 33/53; G01N 33/569

ABSTRACT:

PURPOSE: To enable an antibody to be measured highly sensitively and accurately with freedom from an influence by the storage state of a reagent or the like, regarding a measurement system for an antibody in a specimen with an immunological method based upon a reagent to use antigen having a sensitive thiol group or peptide having action substantially equivalent thereto.

CONSTITUTION: Regarding an antigen having a sensitive thiol group or a reagent containing peptide having action substantially equivalent thereto for immunologically measuring an antibody in a

specimen, the reagent is made to contain a reducing agent, or the antigen having a solid phase of the sensitive thiol group or the reagent containing peptide having action substantially equivalent thereto is treated with the reducing agent. The antibody can thereby be measured highly sensitively and accurately without an influence by the storage state of the reagent or the like. As a result, the sensitivity of the reagent can be enhanced at the time of immunologically measuring the antibody in the specimen.

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Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWOC	Image
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2. Document ID: JP 06074956 A

Entry 2 of 2

File: DWPI

Mar 18, 1994

DERWENT-ACC-NO: 1994-129040

DERWENT-WEEK: 199416

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TITLE: Reagent for antibody determin. esp. of hepatitis C virus -
contg. antigen or peptide with thiol gp., with reagent contg. or
treated with reducing agent

PATENT-ASSIGNEE: DAINABOT CO LTD [DAINN]

PRIORITY-DATA:

1992JP-0270684

August 28, 1992

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
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INT-CL (IPC): G01N 33/53; G01N 33/569; G01N 33/576

ABSTRACTED-PUB-NO: JP06074956A

BASIC-ABSTRACT:

The antibody immunoassays contains an antigen which has a sensitive thiol group or peptide having the same effect; The reagent contains a reducing agent; or the reagent is treated with a reducing agent.

Pref. the reducing agent is antioxidant of thiol group, esp. dithiothreitol, dithioerythritol and/or thioglycolic acid, etc.; The antigen is HCV antigen or the NS3 region of non-structural region of HCV genome; The reagent contains carrier comprising tubes, plates, erythrocytes or latex particles.

By containing a reducing agent in the reagent or by treating the

reagent with a reducing agent, the sensitivity of the reagent for the determination can be raised.

USE/ADVANTAGE - The invention relates to a reagent for the determination of antibody, esp. antibody to hepatitis C virus (HCV). HCV antibody can be accurately determined with high sensitivity.

TITLE-TERMS: REAGENT ANTIBODY DETERMINE HEPATO VIRUS CONTAIN ANTIGEN PEPTIDE THIOL GROUP REAGENT CONTAIN TREAT REDUCE AGENT

DERWENT-CLASS: B04 D16 S03

CPI-CODES: B04-B04C1; B04-G08; B11-C07A; B12-K04A4; D05-H07;

EPI-CODES: S03-E14H4;

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Chemical Indexin g M1 *01* Fragmentation Code M423 M430 M782 M903 N102 P831 Q233 Q505 Chemical Indexing M1 *02* Fragmentation Code M423 M750 M903 N102 Q233 V600 V611 Chemical Indexing M6 *03* Fragmentation Code M903 P831 Q233 Q505 R515 R520 R521 R622 R630

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CPI Secondary Accession Numbers: C1994-059563

Non-CPI Secondary Accession Numbers: N1994-101241

Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWIC	Image
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REAGENT FOR MEASURING ANTIBODY
[Kotai Sokutei Yo Shiyaku]

Toshinori Takei et al.

UNITED STATES PATENT AND TRADEMARK OFFICE
Washington, D.C. May 2000

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Inventors : Toshinori Takei, Yuzo Inoue, Aki
Asahina, and Susumu Tokida

Applicant : Dianabot, Co., Ltd.

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33/53
33/569

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Foreign Language Title : Kotai Sokutei Yo Shiyaku

English Title : REAGENT FOR MEASURING ANTIBODY

Abstract

/1¹

Purpose: To more precisely measure an antibody with high sensitivity without being influenced by storage state, etc., of a reagent in an antibody measurement system in a specimen by an immunological method of the reagent using an antigen having a sensitive thiol group or a peptide having an action substantially equivalent to that of the antigen.

Constitution: In a reagent containing an antigen having a sensitive thiol group for measuring an antibody in a specimen by an immunological method or a peptide having an action substantially similar to that of the antigen, the antibody can be more precisely measured with high sensitivity without being influenced by storage state, etc., of the reagent by including a reducing agent in said reagent or treating a reagent containing an antigen having said sensitive thiol group solidified or a peptide having an action substantially similar to that of the antigen with the reducing agent.

Effect: When the antibody in a specimen is measured by the immunological method, the sensitivity of the reagent can be raised.

Specification

/2

1. Title of the Invention: REAGENT FOR MEASURING ANTIBODY
2. Claims

¹ Numbers in the margin indicate pagination in the foreign text.

1. A reagent for measuring an antibody characterized by the fact that in a reagent containing an antigen having a sensitive thiol group for measuring an antibody in a specimen by an immunological method or a peptide having an action substantially similar to the antigen, a reducing agent is included in said reagent or a reagent containing an antigen having said sensitive thiol group solidified or a peptide having an action substantially similar to that of the antigen is treated with the reducing agent.

2. The reagent for measuring an antibody of Claim 1, wherein said antigen is a HCV antigen or a peptide having an action substantially similar to that of the antigen.

3. The reagent for measuring an antibody of Claim 1, wherein the reducing agent exists in a solvent for reaction.

4. The reagent for measuring an antibody of Claim 1 characterized by the fact that said reagent is an antigen in which a HCV antigen or a peptide having an action substantially equivalent to that of the HCV antigen is solidified; that said reagent is treated with a reducing agent.

5. The reagent for measuring an antibody of Claim 2 or 4, wherein said antigen is a [✓]NS3 area of an unstructured area on a HCV genom.

6. The reagent for measuring an antibody of Claim 5, wherein said antigen is a product manifested by a gene

recombination technique.

7. The reagent for measuring an antibody of Claim 5, wherein said antigen is a synthetic peptide.

8. The reagent for measuring an antibody of Claim 1 characterized by the fact that said reagent includes a carrier; that said carrier is bead, tube, plate, red blood cell, or latex particle.

9. The reagent for measuring an antibody of Claims 1-8, wherein the reducing agent is an antioxidant of a thiol group.

10. The reagent for measuring an antibody of Claims 1-8, wherein the reducing agent is at least one being selected from a group comprised of dithiothreitol, dithioerythritol, thioglycollic acid, cysteine, glutathione, 2-mercaptoethanol, 2-mercaptoethylamine, and these mixture.

3. Detailed explanation of the invention

[0001]

(Industrial application field)

The present invention pertains to a reagent for measuring an antibody, in particular, a reagent for measurement, which more precisely measures an antibody with high sensitivity without being influenced by storage state, etc., of the reagent, in a reagent for measuring an antibody in a specimen by an immunological method using an antigen having a sensitive thiol group or a peptide having an action substantially similar to that

of the antigen. In particular, the present invention pertains to a reagent for measuring an antibody to a C type hepatitis virus (HCV), especially, a reagent for measurement which more precisely measures the HCV antibody in a specimen with high sensitivity.

[0002]

(Prior art)

As a method for diagnosing an infection due to the C type hepatitis virus (HCV), a HCV antibody measurement system using a C100-3 antigen being coded in NS3 and NS4 areas, which are unstructured areas on a HCV genom, was developed in 1988 by US Cairon[transliteration] Co. In 1991, a HCV antibody measurement system with more excellent sensitivity and detection rate with the use of a core antigen and a 33C antigen being coded in a core area, which was a structured area on the HCV genom, and a NS3 area, which was not overlapped with the 100-3 antigen, was developed. As a method for measuring these HCV antibodies, agglutination method using red blood cells or latex particles as an antigen carrying carrier, immunometric method using bead, tube, or plate as an antigen solidifying carrier, etc., are used. However, since the activity of the antigen was rapidly lowered in a reaction solution during the process for solidifying the antigen on the carrier or during the storage of the reagent prepared and the antigen-antibody reaction could not be sufficiently advanced, the measurement sensitivity was not

sufficiently raised. Furthermore, since the activity of the antigen was changed with time, the reproducibility of the sensitivity was deteriorated. In the reagent using such an antigen, the activity of the antigen was rapidly lowered in a reaction solution during the storage of the reagent, and the antigen-antibody reaction could not be sufficiently advanced. Also, the measurement sensitivity was not sufficiently raised, or the activity of the antigen was changed with time, so that the reproducibility of the sensitivity was deteriorated.

[0003]

(Problems to be solved by the invention)

These inventors earnestly researched the problems of the sensitivity in the conventional HCV antibody measurement systems. As a result, it was elucidated that the HCV antigen, in particular, a cysteine included in a protein coded in the NS3 area on the HCV genom was subjected to a natural oxidation and changed to disulfide bond, etc., so that the sensitivity inferiority was caused. Based on these knowledge, these inventors discovered that with the addition of a reducing agent, in particular, a thiol protecting agent to the HCV measurement system, the sensitivity decrease of the HCV antibody measurement system could be prevented and the reproducing agent treatment had no negative influence on said measurement system. From this knowledge, these inventors considered that in the antibody

measurement system in a specimen by an immunological method using a reagent containing an antigen having a sensitive thiol group or a peptide having an action substantially equivalent to that of the antigen, with the addition of the reducing agent, in particular, thiol protecting agent, not only the sensitivity decrease could be prevented, but no negative influence was affected on said measurement system. Then, the present invention was completed.

[0004]

(Means to solve the problems)

The present invention is characterized by the fact that in a reagent containing an antigen having a sensitive thiol group for measuring an antibody in a specimen by an immunological method or a peptide having an action substantially similar to the antigen, a reducing agent is included in said reagent or a reagent containing an antigen having said sensitive thiol group solidified or a peptide having an action substantially similar to that of the antigen is treated with the reducing agent. Thus, the sensitivity of the measurement system is raised, or the reliability of the measurement results is raised. In particular, the present invention is characterized by the fact that in a reagent for measuring a HCV antibody in a specimen by an immunological method, a reducing agent is included in the reagent containing said HCV antigen and a peptide having an action

substantially similar to that of the antigen, or the HCV antigen solidified to a carrier or the peptide having an action substantially similar to that of the HCV antigen is treated with the reducing agent. Thus, the sensitivity of the measurement system is raised, or the reliability of the measurement results is raised. As the specimen being used in the present invention, any components ascribed to the living body may be used, and for example, body fluids or tissue fluids such as blood, serum, semen, spinal fluid, lymph, sputum, tear, saliva, milk juice, leucocyte, digestive organ mucus, and urine are mentioned. Furthermore, in vitro cell culture solutions are mentioned, however the specimen is not limited to these. /3

[0005] In the present invention, in manufacturing a reagent being used to immunologically measure a specific antibody in a specimen by an agglutination reaction, using a sensitive carrier in which an antigen having a sensitive thiol group or a peptide having an action substantially similar to that of the antigen is sensitized to a fine particular carrier such as red blood cell and latex particle, the antigen is sensitized to the carrier, the sensitized carrier obtained is dispersed in a buffer solution containing a reducing agent, and the buffer solution containing the above-mentioned sensitized carrier is freeze-dried. In particular, in the present invention, in manufacturing a reagent being used to immunologically measure the HCV antibody in a

specimen by the agglutination reaction, using a sensitized carrier in which the HCV antigen is sensitized to a fine particle carrier such as red blood cell and latex particle, the HCV antigen is sensitized to a carrier, the sensitized carrier obtained is dispersed in a buffer solution containing a reducing agent, and the buffer solution containing the above-mentioned sensitized carrier is freeze-dried. As the reducing agent usable in the present invention, substances known as a thiol protecting agent are mentioned. Also, as the reducing agent, an antioxidant of a thiol group is preferable, and for example, at least one being selected from a group comprised of dithiothreitol, dithioerythritol, thioglycollic acid, cysteine, glutathione, 2-mercaptoethanol, 2-mercaptoethylamine, and these mixture is mentioned. In particular, dithiothreitol, glutathione, 2-mercaptoethanol, etc., are preferable.

[0006] As the fine particle carrier usable in the present invention, substances broadly known as a carrier for fine particles are mentioned, and for example, in addition to substances made of natural or synthetic high-molecular substances such as synthetic resin and nitrocellulose, latex particles or red blood cells are mentioned. According to the present invention, the above-mentioned reducing agent may also be added into the buffer solution being used in suspending the sensitized carrier during the agglutination reaction. Also, in the present

invention, in manufacturing a reagent being used to immunologically measure a specific antibody in a specimen by methods such as enzymeimmunoassay (EIA), radioimmunoassay (RIA), or fluoroimmunoassay (FIA), using a solidified antigen obtained by solidifying an antigen having a sensitive thiol group or a peptide having an action substantially similar to that of the antigen upon an insoluble carrier, the antigen having a sensitive thiol group or the peptide having an action substantially similar to that of the antigen is solidified upon the insoluble carrier. Then, the antigen-bonded solid phase obtained is immersed into a buffer solution containing the above-mentioned reducing agent or these mixture, and the above-mentioned solid phase is dried. In particular, in the present invention, in manufacturing a reagent being used to immunologically measure a HCV antibody in a specimen by methods such as enzymeimmunoassay (EIA), radioimmunoassay (RIA), or fluoroimmunoassay (FIA), using the solidified antigen obtained by solidifying the HCV antigen upon the insoluble carrier, the antigen-bonded solid phase obtained is immersed into a buffer solution having the above-mentioned reducing agent or these mixture, and the above-mentioned solid phase is dried.

[0007] As the reducing agent usable in the present invention, substances known as a thiol protecting agent similar to the above-mentioned one are mentioned. According to the present

invention, the above-mentioned reducing agent or these mixture may also be added into a reaction solution being used in reacting a specific antibody in a specimen with the antigen-bonded solid phase. As the insoluble carrier usable in the present invention, substances broadly known as a carrier for a solid phase process are mentioned, and for example, there are synthetic resin such as polyethylene, polypropylene, polystyrene, and polyacrylate, natural or synthetic high-molecular substances such as nitrocellulose, polymerized amino acid, and polysaccharide, particles, films, beads, tubes, or plates made of glasses. Also, in the solid phase process, there is a physical adsorbing method or a chemically solidifying method using a binder. The chemical binder can be selected from ordinary substances known to the party concerned, and for example, activated ester of 6-maleimidecaproic acid, 2-bromoacetic acid, 2-iodoacetic acid, succinic acid, etc., activated ester of triazine, sulfonic ester derivative, etc., are mentioned. However, the present invention is not limited to these.

[0008] As the antigen being used in the present invention, an antigen having a sensitive thiol group or a peptide having an action substantially similar to that of the antigen is mentioned. Here, the sensitive thiol group is a thiol group in cysteine included in a protein or peptide and means a group that is sensitive to a natural oxidation or artificial oxidation under an

ordinary condition and can have a large influence on the activation of said antigen. In particular, it indicates the activation of a specific antigen being reacted with a specific antibody in a specimen by an immunological method. As the antigen being used in the present invention, a recombinant antigen being a manifested product prepared by a genetic engineering method or a synthetic peptide can be used without a particular limitation as long as it has a sensitive thiol group. In the present invention, even if the thiol group of cysteine or disulfide bond ascribed to it is included in the molecule, if they are not groups that can have an influence on the activation of said antigen, they are not used as the antigen for the treatment with a reducing agent in the present invention. In particular, as the HCV antigen being used in the present invention, a recombinant HCV antigen being a manifested product prepared by a genetic engineering method or a HCV antigen peptide which is a synthetic peptide is mentioned. As the HCV antigen being used in the present invention, a HCV antigen corresponding to NS3 area of an unstructured area on the HCV genom is preferably mentioned.

/4

[0009]

(Application examples)

Next, the present invention is explained in further detail by application examples.

Application Example 1: Influence of DTT addition on sensitized blood cells

Human immobilized red blood cells were washed three times with a phosphoric acid buffer solution (pH 7.4) and suspended at 1 vol% in an acetic acid buffer solution of pH 5.7, and a refined HCV antigen (core antigen, a mixture of 33C antigen and 100 antigen) being produced by a gene recombination technique was added to it so that the final concentration could be 6 $\mu\text{g/ml}$. Then, they were stirred for 1 h in a greenhouse, washed three times with a phosphoric acid buffer solution (pH 7.4), and freeze-dried in a phosphoric acid buffer solution (pH 7.4) containing 7.5% sucrose, so that HCV antigen-sensitized red blood cells were prepared. The HCV antigen-sensitized red blood cells obtained were re-suspended at 1 vol% in a tris hydrochloric acid buffer solution (pH 7.8) containing 2 mM dithiothreitol (DTT), and for comparison, said red blood cells were suspended in a tris hydrochloric acid buffer solution containing no DTT. Then, their sensitivity was compared. In the comparison of the sensitivity, a HCV antibody-positive human serum was diluted with a serum in which said antibody was negative, at a prestage and used as a serum for controlling the sensitivity. In each well of a microtiter plate, 25 μl phosphoric acid buffer solution and 25 μl serum for controlling the sensitivity at each concentration were poured, and 25 μl sensitized blood cells was poured. Then,

they were mixed for 30 sec by a mixer and held at room temperature for 1 h, and the results were decided with the naked eyes.

(Table I)

	Sensitized blood cell suspension	
	Existence of DDT	Absence of DDT
Dilution rate of the serum for controlling the sensitivity		
Serum A		
X 1	+	+
X 2	+	+
X 4	+	+
X 8	+	-
X16	-	-
X32	-	-
X64	-	-
Serum B		
X 1	+	+
X 2	+	+
X 4	+	+
X 8	+	-
X16	-	-
X32	-	-
X64	-	-

(+: positive, -: negative)

From the results of Table I, it was shown that when the sensitized blood cells were re-suspended in the buffer solution containing DTT, the sensitivity was raised.

[0010]

Application Example 2: Influence of 2-ME addition on sensitized blood cells

Sensitized blood cells prepared similarly to Application

Example 1 were re-suspended at 1 vol% in a tris hydrochloric acid buffer solution (pH 7.8) containing 40 mM 2-mercaptoethanol (2-ME), and as for the storage stability after the suspension, a tris hydrochloric acid buffer solution containing no 2-ME was compared. The storage after dissolving was carried out at 2-8°C, and the sensitivity of the sensitized blood cells was compared according to Application Example 1.

(Table II)

/5

① 2~8℃ における 保存期間 (日)	② 感作血球懸濁液					
	③ 2-ME存在			④ 2-ME非存在		
	0	7	14	0	7	14
⑤ 感度管理用 血清の希釈率						
⑥ 血清A						
× 1	+	+	+	+	+	+
× 2	+	+	+	+	+	-
× 4	+	+	+	+	-	-
× 8	+	+	+	+	-	-
× 16	-	-	-	-	-	-
× 32	-	-	-	-	-	-
× 64	-	-	-	-	-	-
⑦ 血清B						
× 1	+	+	+	+	+	+
× 2	+	+	+	+	+	-
× 4	+	+	+	+	-	-
× 8	+	+	+	+	-	-
× 16	-	-	-	-	-	-
× 32	-	-	-	-	-	-
× 64	-	-	-	-	-	-

⑧ (+ : 陽性、- : 陰性)

- [1. Storage period (day) at 2-8°C
2. Sensitized blood cell suspension
3. Existence of 2-ME

4. Absence of 2-ME
5. Dilution rate of the serum for controlling the sensitivity
6. Serum A
7. Serum B
8. (+: positive, -: negative)]

From the results of Table II, it was confirmed that with the addition of 2-ME, the storage stability after suspending the sensitized blood cells was improved.

[0011]

Application Example 3: Influence of glutathione addition on sensitized blood cells

Blood cells, in which a HCV antigen was sensitized according to Application Example 1, were freeze-dried in a buffer solution for freeze-drying in which glutathione (GSH) was added to a phosphoric acid buffer solution (pH 7.4) containing 7.5% sucrose so that the final concentration could be 40 mM, so that sensitized blood cells were obtained. For comparison, the blood cells were freeze-dried in a buffer solution containing no GSH, so that comparative sensitized blood cells were obtained. The sensitized blood cells and the comparative sensitized blood cells were suspended at 1 vol% in a tris hydrochloric acid buffer solution (pH 7.8), and the stability after suspending at room temperature was compared by the sensitivity of the sensitized blood cells. The sensitivity was compared according to

Application Example 1.

(Table III)

/6

① 室温での 保存期間 (日)	② 凍結乾燥用緩衝液					
	③ GSH存在			④ GSH非存在		
	0	1	2	0	1	2
⑤ 感度管理用 血清の希釈率						
⑥ 血清A						
× 1	+	+	+	+	+	+
× 2	+	+	+	+	+	-
× 4	+	+	+	+	-	-
× 8	+	+	+	+	-	-
× 16	-	-	-	-	-	-
× 32	-	-	-	-	-	-
× 64	-	-	-	-	-	-
⑦ 血清B						
× 1	+	+	+	+	+	-
× 2	+	+	+	+	+	-
× 4	+	+	+	+	-	-
× 8	+	+	+	+	-	-
× 16	-	-	-	-	-	-
× 32	-	-	-	-	-	-
× 64	-	-	-	-	-	-

⑧ (+ : 陽性、- : 陰性)

1. Storage period (day) at room temperature
2. Buffer solution for freeze-drying
3. Existence of GSH
4. Absence of GSH
5. Dilution rate of the serum for controlling the sensitivity
6. Serum A
7. Serum B
8. (+: positive, -: negative)]

From the results of Table III, it was confirmed that the sensitized blood cells freeze-dried by adding the GSH had favorable stability after suspending.

[0012]

Application Example 4: Influence of 2-ME addition on solidified antigen

Polystyrene beads of 1/4 inch were put into a phosphoric acid buffer solution (pH 7.4) containing 7.5% sucrose, and the HCV antigen used in Application Example 1 was added to it so that the final concentration could be 10 μ l. Then, they were held at 37°C for 1 h, washed three times with a phosphoric acid buffer solution (pH 7.4), immersed into a phosphoric acid buffer solution (pH 7.4) containing 7.5% sucrose, and dried, so that HCV antigen-solidified beads were obtained. One piece of the above-mentioned HCV antigen-solidified beads was put into a tray of a wide well, in which 200 μ l tris hydrochloric acid buffer solution (pH 7.8) containing 20 mM 2-ME was included as a buffer solution for reaction, and held at room temperature for 1 h. 10 μ l serum for controlling the sensitivity was added to it and reacted at 37°C for 1 h. After finishing the reaction, it was washed three times with a physiological saline solution, and 200 μ l peroxidase mark antihuman immunoglobulin G antibody was added to it and reacted at 37°C for 30 min. After finishing the reaction, it was washed three times with a physiological saline solution, and a

matrix solution containing orthoethylenediamine and hydrogen peroxide was added at 1 ml and reacted at room temperature for 30 min. Then, for decision, the absorbance at 490 nm was measured. For comparison, using a tris hydrochloric acid buffer solution containing no 2-ME, a similar operation was carried out and decided.

(Table IV)

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① 測定用緩衝液					
② 2-ME 存在			③ 2-ME 非存在		
吸光度 ④ 判定			吸光度 ⑤ 判定		
⑥ 感度管理用血清の希釈率					
⑦ 血清A					
×	1	1. 8 2	+	0. 6 9	+
×	2	1. 4 4	+	0. 4 5	-
×	4	0. 9 6	+	0. 3 2	-
×	8	0. 5 6	+	0. 2 6	-
⑧ 血清B					
×	1	1. 2 9	+	1. 3 1	+
×	2	0. 9 3	+	0. 7 3	+
×	4	0. 5 9	+	0. 4 7	-
×	8	0. 3 8	-	0. 3 0	-
×	16	0. 2 5	-	0. 2 6	-

⑨ (+ : 陽性、- : 陰性)

- [1. Buffer solution for measurement
2. Existence of 2-ME
3. Absence of 2-ME
4. Absorbance decision
5. Absorbance decision
6. Dilution rate of the serum for controlling the sensitivity

7. Serum A

8. Serum B

9. (+: positive, -: negative)]

From the results of Table IV, it was confirmed that the increase of the sensitivity was recognized by adding 2-ME to the buffer solution for reaction.

[0013] Application Example 5: Influence of 2-ME addition

HCV antigen-sensitized blood cells prepared according to Application Example 1 were suspended in a tris hydrochloric acid buffer solution (pH 7.8) and held at 2-8°C for 2 weeks, and as HCV antibody-positive specimens, as for three kinds of specimens mainly containing each antibody of anticore (core are of HCV), anti-33C (NS3 area of HCV), and anti-C100 (NS3-NS4 area of HCV), the sensitivity of the sensitized blood cells was measured and reviewed according to Application Example 1. 40 mM 2-ME was added to a suspension, and the sensitivity was similarly reviewed.

(Table V)

	① 懸濁液に2-MEを 添加した感作血球	② 懸濁液に2-MEを 添加しない感作血球
③ 検体の希釈率		
④ 抗33C抗体		
× 128	+	+
× 256	+	+
× 512	+	-
× 1024	+	-
× 2048	+	-
× 4096	+	-
× 8192	-	-

⑤ (+: 陽性, -: 陰性)

- [1. Sensitized blood cells in which 2-ME was added to the suspension
2. Sensitized blood cells in which 2-ME was not added to the suspension
3. Dilution rate of the specimens
4. Anti-33C antibody
5. (+: positive, -: negative)]

From these results, it was recognized that the sensitivity of the sensitized blood cells, whose sensitivity was decreased by holding for 2 weeks, was recovered with the addition of 2-ME in the 33C antigen being coded in NS3 area on the HCV genom.

[0014]

(Effects of the invention)

In including a reducing agent in a reagent for measuring an antibody containing an antigen having a sensitive thiol group or a peptide having an action substantially similar to that of the antigen or treating said solidified reagent with the reducing agent and measuring the antibody in a specimen by an immunological method, the sensitivity of the reagent can be raised.